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Brief Report

(D-Ser2)Oxm[Lys38-γ-glu-PAL] improves hippocampal gene expression and cognition in a mouse model of type 1 diabetes

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ABSTRACT

Objective: Oxyntomodulin (Oxm) is a gastrointestinal hormone with recently noted therapeutic potential for type 1 diabetes mellitus (T1DM). The present study examined the effects of a stable Oxm analogue on anxiety, exploratory behavior, cognitive function, hippocampal gene expression and metabolic control in a mouse model of T1DM. **Methods:** Effects of twice daily administration of the stable Oxm analogue, (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL], was assessed in insulin-deficient streptozotocin (STZ)-induced T1DM mice. **Results:** Induction of diabetes by STZ injection significantly ($P < 0.05$) impaired learning and memory compared to normal control mice. However, (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] treatment completely reversed this detrimental effect. Anxiety levels and exploratory behavior were not significantly different between all groups of mice. Hippocampal gene expression of MASH1, SYP and mTOR were reduced ($P < 0.01$ to $P < 0.001$) in T1DM mice, but significantly ($P < 0.05$ to $P < 0.001$) enhanced by twice daily (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] intervention. Moreover, expression of SYP, mTOR and IRS-1 were significantly elevated ($P < 0.05$ to $P < 0.001$) in (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] mice compared to both STZ and lean controls. These effects were accompanied by improved ($P < 0.001$) glucose tolerance and insulin sensitivity compared to STZ controls. **Conclusion:** The data highlight the potential of (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] for the treatment of T1DM, and reveal the ability of this Oxm analogue to restore the deficits of learning and memory observed in STZ-induced T1DM.

KEY WORDS: Oxyntomodulin (Oxm); Type 1 diabetes mellitus (T1DM); Cognition; Hippocampus

INTRODUCTION

Oxyntomodulin (Oxm) is a proglucagon derived gut peptide secreted by enteroendocrine L-cells in response to feeding, which is known to activate both glucagon and GLP-1 receptors [1]. Accumulating evidence suggests that stable Oxm analogues represent an attractive potential therapeutic option for obesity and related metabolic disease [2]. As such, numerous studies confirm that through glucagon receptor activation, Oxm induces catabolic effects that favor weight loss, while glucose homeostasis is improved through activation of GLP-1 receptors [1]. To capitalize on this therapeutic profile, we have recently developed (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL], an enzymatically stable Oxm analogue, with a significantly a protracted *in vivo* biological action profile [3].

Despite a clear for use of stable Oxm analogues for the treatment of type 2 diabetes, effectiveness in insulin-deficient type 1 diabetes mellitus (T1DM) is unknown. Thus, it might seem implausible that up-regulation of glucagon receptor signaling would be advantageous in T1DM. However, activation of GLP-1 receptors on both alpha- and beta-cells by Oxm would impart beneficial intraislet-cell effects in T1DM [4]. Indeed, we have recently shown that sustained treatment with (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] improves metabolic control and islet morphology in an experimental model of T1DM [5]. However, further observations from our laboratory reveal that Oxm-mediated actions are not limited to the pancreas and gut, and that Oxm crosses the blood brain barrier [2]. As such, activation of Oxm signaling pathways in the hippocampal brain regions in an animal model of type 2 diabetes was associated with positive effects on cognitive function and overall learning and memory processes [2]. Therefore, we have now examined the consequence of twice daily (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] administration on metabolic status, anxiety, exploratory behavior, cognitive function and the expression of key hippocampal genes involved in learning and memory in insulin-deficient streptozotocin (STZ)-induced T1DM mice.

MATERIALS AND METHODS

Peptides

(D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] was purchased from GL Biochem Ltd. (Shanghai, China) and characterized as described previously [3].

Animals

Adult male NIH mice (14-16 weeks old; Harlan UK Ltd, Blackthorn, UK) were housed individually in an air-

conditioned room at 22 ± 2°C with 12:12 h light/dark cycle. Mice had free access to drinking water and standard rodent maintenance diet that contained 10% fat, 30% protein and 60% carbohydrate (Trouw Nutrition, Cheshire, UK). Diabetes was induced by intraperitoneal (i.p.) injection of 12 h fasted mice with 150 mg/kg streptozotocin (STZ) freshly prepared in ice cold 0.1 M sodium citrate buffer, pH 4.5. All animal procedures were carried out according to the UK home office regulations (UK Animal Scientific Procedures Act 1986).

In vivo studies

Following STZ administration, mice (n=8) received twice daily injections (08:00 and 17:00 h) of saline vehicle (0.9% (w/v) NaCl) or (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] (25 nmol/kg, b.w.) for 28 days. All STZ injected mice received insulin (15 U/kg b.w. once daily, bovine insulin, Sigma-Aldrich, Poole, UK) for the first 5 days of the study. Insulin therapy was only maintained thereafter in diabetic control mice. Normal mice maintained on standard diet, without STZ intervention, and treated twice daily with saline were used for comparative purposes. Body weight, non-fasting plasma glucose and insulin concentrations were recorded on day 28. In addition, i.p. glucose tolerance (18 mmol/kg body wt) and insulin sensitivity (20 U/kg body wt) tests were performed at the end of the study period.

Open field assessment and object recognition task

For open field assessment, at the end of the study mice were placed in an exploratory arena (58 cm diameter, 38 cm high) for 5 minute and a computerized tracking system (Biosignals, New York) analyzed measures of speed, distance travelled, rearing actions (indicator of exploratory activity) and grooming events (indicator of anxiety levels) [6]. For object recognition, animals were placed in the same arena and two identical random objects (2 marbles, 2.5 cm diameter; or 2 dice, 1.2 cm side length) were positioned in the center of the arena (dimensions outlined above). Four hours after initial exposure (the acquisition phase), one of the two objects was replaced by a novel object (a marble or dice) and the time spent exploring both objects during a 5 minute trial phase determined. Recognition index (RI) was calculated as described previously [6].

Hippocampal gene expression

Whole hippocampus tissue was excised at the end of the treatment period, snap frozen and processed for gene expression by qPCR following total RNA extraction (Tripure Isolation Reagent; Roche Diagnostics, West Sussex, UK). cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Gene expression analysis was carried out using a Roche Real Time ready qPCR assay and Light Cycler 480 Probes Master and a hot start reaction mix (Roche Diagnostics, West Sussex, UK), according to the Manufacturer's instructions. The following target genes were designed and supplied by Roche (Roche Probe master): insulin receptor substrate-1 (IRS-1), synaptophysin (SYP); mammalian target of rapamycin (mTOR) and mammalian achaete-scute homologue 1 (MASH1). Gene expression was normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) expression and relative quantification assessed using the $2^{-\Delta\Delta CT}$ method to calculate differences in gene expression between samples, as described previously [2].

Statistical analysis

Results are presented as mean ± SEM. Groups of data were compared using ANOVA and unpaired Student's t-test in GraphPad PRISM (version 3.0). Differences were considered significant if $P < 0.05$.

RESULTS

Effects of (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] on metabolic control in STZ-induced diabetic mice

Twice daily treatment with (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] resulted in the complete normalization of body weight in STZ-induced diabetic mice by day 28 (Table 1). Non-fasting blood glucose and plasma insulin levels were still increased ($P < 0.001$) and decreased ($P < 0.001$), respectively, compared to normal control mice, but they were significantly improved ($P < 0.01$ and $P < 0.001$, respectively) compared to STZ diabetic controls (Table 1), despite continued insulin therapy in STZ control mice. AUC glucose tolerance values were significantly ($P < 0.001$) improved compared to STZ controls, but elevated ($P < 0.001$) when compared to normal control mice (Table 1). In addition, the hypoglycemic action of insulin was significantly ($P < 0.05$) augmented in (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] treated mice compared to STZ diabetic controls (Table 1).

Effects of (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] on anxiety levels, exploratory behavior and hippocampal gene expression

Open field assessment revealed no effect of 28 days twice daily treatment with (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] on the number of grooming (anxiety level) and rearing (exploration) episodes, as well as distance travelled and average speed, when compared to STZ and lean control mice (data not shown). However, STZ diabetic control mice had significantly ($P < 0.05$) impaired recognition memory by the end of the study, which was completely restored by (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] treatment (Figure 1A, B). Indeed, (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] mice displayed a similar preference to explore the novel object as normal control mice (Figure 1B).

Table 1. Effects of (d-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] on metabolic parameters in STZ-induced diabetic mice

Parameter	STZ diabetic control	(d-Ser^2)Oxm[Lys^{38}-γ-glu-PAL]	Lean control
Body weight (g)	16.0 ± 0.2***	19.5 ± 0.3$^{\Delta\Delta\Delta}$	20.5 ± 0.3
Non-fasting glucose (mmol/l)	34.5 ± 1.1***	25.7 ± 1.1***,$^{\Delta\Delta}$	3.6 ± 0.2
Non-fasting insulin (ng/ml)	0.1 ± 0.1***	0.6 ± 0.1***,$^{\Delta\Delta\Delta}$	1.1 ± 0.1
Glucose tolerance: 0-60 min glucose AUC (mmol/l.min)	705.7 ± 47.0***	526.7 ± 19.0***,$^{\Delta\Delta\Delta}$	331.1 ± 18.4
Insulin sensitivity: glucose 0-60 min AAC (mmol/l.min)	412.5 ± 67.2	808.2 ± 95.9$^{\Delta}$	530.7 ± 179.0

Parameters were measured after 28 days treatment with saline vehicle or (d-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] (25 nmol/kg). For glucose tolerance, glucose (18 mmol/kg body wt) was administered in non-fasted mice and 0-60 min plasma glucose AUC values calculated. For insulin sensitivity, insulin (20 U/kg body wt) was administered in non-fasted mice and 0-60 min AAC values calculated. Values are mean ± SEM for six mice. ***P < 0.001 compared to normal lean controls. $^{\Delta}P$ < 0.05, $^{\Delta\Delta}P$ < 0.01, $^{\Delta\Delta\Delta}P$ < 0.001 compared to STZ diabetic controls maintained on insulin (15 U/kg b.w. once daily) throughout.

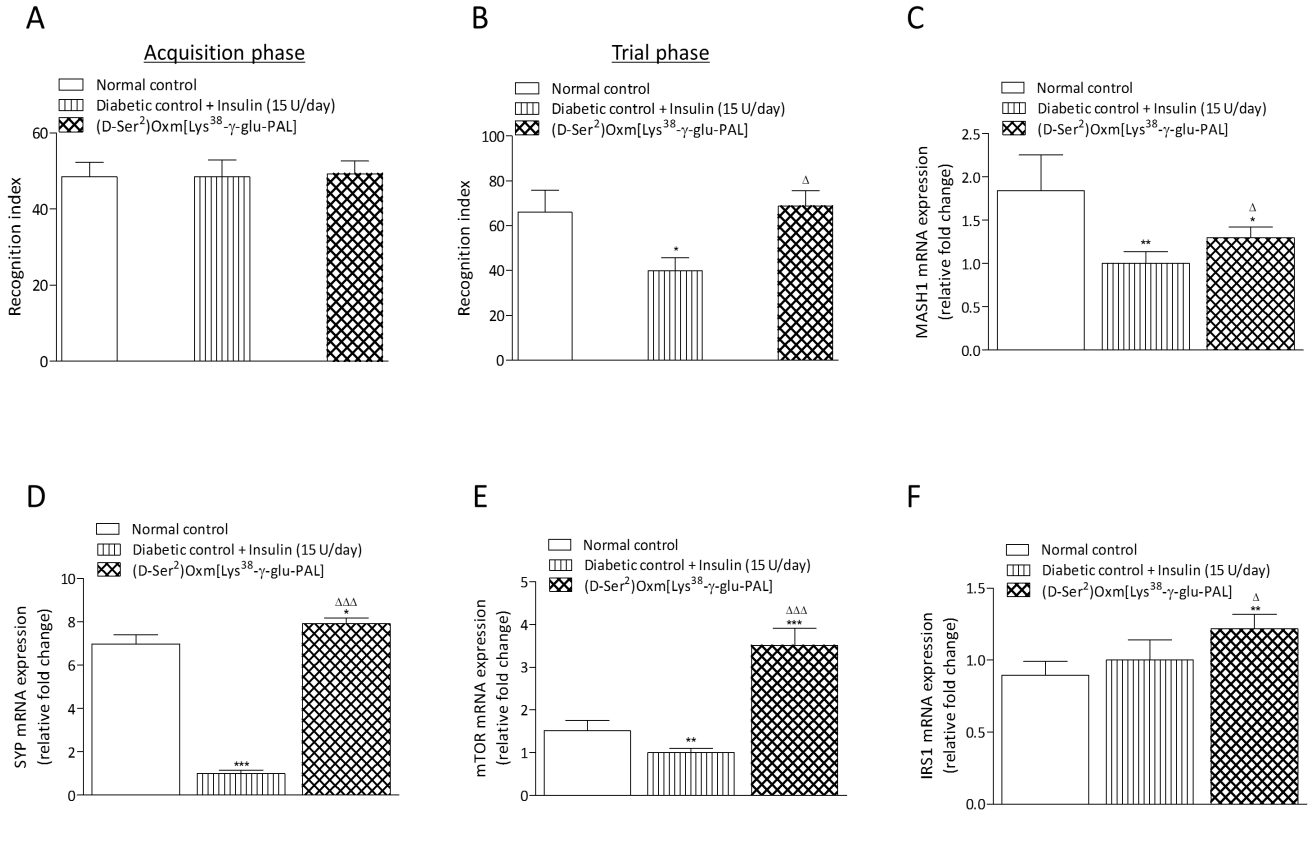

Figure 1. Effects of twice daily (d-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] administration on (A, B) recognition index (RI) and (C-F) hippocampal gene expression in STZ-induced diabetic mice. (A, B) Recognition index (RI) was assessed during the acquisition (A) and test (B) tasks in mice. RI was defined as the amount of time exploring the familiar (tA) or novel object (tB) over the total time spent exploring both objects x 100: (tA or tB/(tA+tB))∗100. (C-F) mRNA expression of (C) MASH1, (D) SYP, (E) mTOR and (F) IRS-1 was examined in the hippocampus and expression normalized to levels of the internal control gene HPRT. All values are mean ± SEM for 5-7 mice. *P < 0.05, **P < 0.01, ***P < 0.001 compared to normal controls. $^{\Delta}P$ < 0.05, $^{\Delta\Delta\Delta}P$ < 0.001 compared to STZ diabetic controls.

Effects of (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] on hippocampal gene expression

Induction of diabetes by STZ injection significantly (P < 0.01 to P < 0.001) reduced hippocampal gene expression of MASH1, SYP and mTOR (Figure 1C-E). (D-Ser^2) Oxm[Lys^{38}-γ-glu-PAL] treatment significantly (P < 0.05) increased MASH1 expression compared to STZ diabetic control mice, although this was still lower (P < 0.05) than normal controls (Figure 1C). However, SPY and mTOR hippocampal gene expression was enhanced compared to both normal (P < 0.05 and P < 0.001; respectively) and diabetic (P < 0.001 in both cases) control mice (Figure 1D,E). STZ diabetic mice had unaltered IRS-1 hippocampal gene expression, but treatment with (D-Ser^2)

Oxm[Lys^{38}-γ-glu-PAL] increased expression compared to normal ($P < 0.01$) and diabetic ($P < 0.05$) control mice (Figure 1F).

DISCUSSION

Consistent with previous observations, up-regulation of Oxm signaling pathways resulted in marked beneficial effects in STZ-induced T1DM mice [4,5]. Thus, twice daily administration of (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] markedly improved glucose homeostasis and insulin sensitivity. Notably, normalization of body weight was also observed in the current study, together with substantially reduced circulating glucose and increased insulin concentrations. We have already shown that these beneficial Oxm-mediated effects are associated with improvements of islet morphology [5]. However, whilst encouraging effects of (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] on cognition have recently been evidenced in type 2 diabetes [2], information in this regard is lacking in insulin-deficient T1DM. In view of the increasing awareness of cognitive defects in diabetes [7], we therefore examined the impact of (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] treatment on anxiety, exploratory behavior, recognition memory and the expression of key hippocampal genes involved in cognition in STZ-induced T1DM mice. In addition, recent evidence suggests a neuroprotective effect of Oxm in a mouse model of Parkinson's disease [8].

In harmony with findings in high fat fed mice [2], treatment with (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] resulted in a marked increase in hippocampal SYP gene expression in STZ-induced T1DM mice. This could point towards improved neuronal communication (synaptogenesis) in these mice [9], as expression was significantly elevated compared to both STZ-diabetic and lean control mice. In accordance with this, expression of MASH1, a molecule important for neuronal growth [10], was also enhanced in the hippocampus of (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] treated STZ mice. As such, augmented MASH1 signaling indicates proliferation and differentiation of hippocampal progenitor neuronal cells [11]. Interestingly, sustained activation of GLP-1 receptors has also been shown to elevate hippocampal mRNA expression of MASH1 in mice [12]. Taken together, these observations suggest improvements in the processes underlying hippocampal-mediated cognitive performance. Thus, (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] treated mice exhibited significantly increased learning and recognition memory, as evidenced by the novel object recognition behavioral task. Moreover, despite daily insulin therapy, STZ diabetic mice exhibited decreased recognition memory, highlighting the clear advantages of (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] therapy in T1DM [5]. Importantly, improved memory was not associated with changes in anxiety or exploratory behavior, which could have otherwise impacted upon these findings.

There is a recognized connection between the development of cognitive decline and insulin resistance [13]. Treatment with (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] resulted in increased hippocampal mRNA expression of IRS-1, an important signaling protein responsible for initiating insulin and insulin-like growth factor signaling pathways [14]. As such, defective IRS-1 dependent insulin signaling in the hippocampus has been associated with severe cognitive deterioration [15]. Interestingly, hippocampal expression of mTOR, a protein kinase involved in the insulin signaling pathway [16], was prominently enhanced by (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] treatment. These observations suggest that the improvement of peripheral insulin sensitivity noted with (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] also extends to its central actions. However, additional assessments of protein expression would also be important to more closely mimic a functional physiological effect for the genes described in this study.

In conclusion, the present study indicates that the therapeutic efficacy of (D-Ser^2)Oxm[Lys^{38}-γ-glu-PAL] in T1DM extends to enhancement of hippocampal signaling pathways involved in cognition and memory function [2]. The extent to which improvements in metabolic control contribute to these effects requires further clarification [17,18], but this study clearly highlights the potential of stable analogues of Oxm as novel therapeutic agents for alleviation of cognitive defects in T1DM.

DECLARATION OF INTEREST

The authors report no conflict of interests.

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Conflict of Interest: None declared